International Immunology Advance Access published August 16, 2004

International Immunology 2004; 1 of 8
doi:10.1093/intimm/dxh147

Isolation of a novel KIR2DL3-specific mAb: comparative analysis of the surface distribution and function of KIR2DL2, KIR2DL3 and KIR2DS2

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Keywords: HLA-specific receptors, immunoglobulin superfamily, KIR, NK cells

Abstract

In recent years an increasing number of sequences coding for new KIRs have been described. However, the limited availability of mAbs with unique KIR specificities has hindered an exhaustive assessment of their actual function, HLA-specificity, expression at the cell surface and distribution in different cell populations. In this study we report the generation of a novel mAb (ECM41) specific for KIR2DL3 molecules. By the use of cell transfectants expressing one or other KIR we show that this reagent allows discrimination of KIR2DL3 from other GL183 mAb-reactive molecules such as KIR2DL2 and KIR2DS2. Moreover we show that this novel mAb can be used to assess the surface expression and distribution of KIR2DL3 in different polyclonal NK populations and in NK cell clones. Along this line, we were able to analyze the HLA class I specificity of NK clones expressing either KIR2DL3 or KIR2DL2, two inhibitory receptors that were so far serologically undistinguishable. Finally, the combined use of GL183 and ECM41 mAbs in redirected killing assays allowed us to investigate the functional outcome of the simultaneous engagement of KIR2DL3 and KIR2DS2 in NK cell clones co-expressing KIRs that display opposite (inhibitory vs activating) function.

Introduction

The NK cell effector function is finely modulated by a series of different surface receptors able to transduce either positive or negative signals (1). In humans, the activating receptors are represented by an array of different receptors and co-receptors including the Natural Cytotoxicity Receptors (NCR) (i.e. NKp46, NKp30 and NKp44) (2), NKG2D (3–5), 2B4 (6–8), NTB-A (9,10), NKp80 (11), CD59 (12) and DNAM 1 (13,14). The main inhibitory receptors include the Killer Ig-like Receptors (KIRs) (15–17), the CD94–NKG2A heterodimer (18,19) and LIR 1/ILT2 (20–22). In particular, KIRs represent a family of clonally distributed receptors, each specific for a different group of HLA class I alleles (15). In their inhibitory forms, KIRs are characterized by the presence of two or three extracytoplasmic Ig-like domains (KIR2D or KIR3D) and transduce the inhibitory signal through the phosphorylation of Immuno Tyrosine-based Inhibitory Motifs (ITIM) inserted in their long cytoplasmic tail (KIR2DL or KIR3DL) (15,17,23). Through NCRs, NKG2D and the various co-receptors NK cells can recognize and kill NK-susceptible target cells while inhibitory KIRs, through their interaction with self HLA class I molecules on target cells, prevent NK cells from killing normal cells. Thus the expression of appropriate HLA-specific inhibitory receptors (i.e. specific for given self HLA class I allele) on NK cells is a necessary requirement to avoid the indiscriminate attack of normal tissues by NK cells. In this context, it should be noted that in recent years an increasing number of sequences coding for novel KIRs have been identified (24–26). However, due to the frequent lack of mAbs with unique KIR specificities, the actual surface expression, distribution and HLA-specificity of these molecules remains incompletely solved.

Interestingly, beyond the inhibitory KIRs, NK cells can also express activating KIRs (15,27,28). These molecules are

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Transmitting editor: E. Vivier

Received 2 July 2004, *accepted* 21 July 2004

characterized by a short cytoplasmic tail (KIR2DS or KIR3DS) that lacks inhibitory motifs and associates to KARAP/DAP12 polypeptides (29,30). Notably, KARAP contains ITAM that are involved in the transduction of triggering signals. Some of the activating KIRs display a high degree of homology with the correspondent inhibitory KIR and at least in some cases have also been shown to recognize (although with lower efficiency) the same HLA class I alleles (25,31,32). Given the high degree of homology between different KIRs, most of the KIR-specific mAbs recognize epitopes shared not only by two or more inhibitory KIRs but also by their activating counterpart. For example no mAbs are available to discriminate KIR2DL1 (inhibitory) from KIR2DS1 (activating) (both reactive with the EB6 or EB6-like mAbs) (15,33); or KIR2DL2 (inhibitory) from KIR2DL3 (inhibitory) and from KIR2DS2 (activating) (all reactive with the GL183 or GL183-like mAbs) (15,33).

To gain further information on the functional relationship between activating and inhibitory KIRs, it would be useful to obtain mAbs allowing to distinguish, for example, the inhibitory form of a given KIR from its activating counterpart. This would enable us to establish at the single cell level which ones among the different KIRs recognized by a given mAb are actually expressed. Importantly, such mAbs would also allow the functional analysis of KIRs whose activating and inhibitory forms might be co-expressed at the surface of the same NK cell.

In this study we describe a novel mAb (termed ECM41) able to discriminate the KIR2DL3 molecule from the other two GL183 mAb reactive KIRs (KIR2DL2 and KIR2DS2). By the combined use of ECM41 and GL183 mAbs, we were able to analyze the expression, the distribution and the HLA-specificity of two inhibitory receptors: KIR2DL3 and KIR2DL2, that were so far serologically indistinguishable. We were also able to analyze the functional cross-talk between KIR2DL3 and KIR2DS2 in NK cells co-expressing these two previously indistinguishable GL183-reactive molecules.

Methods

Monoclonal antibodies

The following mAbs were produced in our lab: JT3A (IgG2a, anti-CD3), BAB281 (IgG1, anti-NKp46), c127 (IgG1, anti-CD16), c218 (IgG1, anti-CD56), A6/136 (IgM, anti-HLA class I), EB6b (IgG1, anti-KIR2DL1/CD158a and KIR2DS1/CD158h), GL183 (IgG1, anti-KIR2DL2/CD158b1, KIR2DL3/CD158b2 and KIR2DS2/CD158j), DF200 (IgG1, anti-KIR2DL1, KIR2DS1, KIR2DL2, KIR2DL3 and KIR2DS2), FES172 (IgG2a, anti-KIR2DS4/CD158i), Z27 (IgG1, anti-KIR3DL1/CD158e1 and KIR3DS1/CD158e2), Q66 (IgM, anti-KIR3DL2/CD158k), AZ158 (IgG2A, anti-KIR3DL1 and KIR3DL2) (15). D1.12 (IgG2a, anti-HLA-DR) mAb was provided by Dr R.S. Accolla (Pavia, Italy). HP2.6 (IgG2a, anti-CD4) mAb was provided by Dr P. Sanchez (Madrid, Spain).

Purification of peripheral blood lymphocytes (PBL) and generation of polyclonal or clonal NK cell populations

Peripheral blood lymphocytes (PBL) were derived from healthy donors by Ficoll–Hipaque gradients and depletion of plastic-adherent cells. In order to obtain purified NK cells, PBL were incubated with anti-CD3 (JT3A), anti-CD4 (HP2.6) and anti-HLA-DR (D1.12) mAbs (30 min at 4°C) followed by goat anti-mouse coated Dynabeads (Dynal, Oslo, Norway) (30 min at 4°C) and immunomagnetic depletion (33). $CD3^-4^-DR^-$ cells were cultured on irradiated feeder cells in the presence of 100 U/ml rIL-2 (Proleukin, Chiron Corp., Emeryville, USA) and 1.5 ng/ml PHA (Gibco Ltd, Paisley, Scotland) in order to obtain polyclonal NK cell populations or, after limiting dilution, NK cell clones.

Flow cytofluorimetric analysis

Cells were stained with the appropriate mAb followed by PE- or FITC-conjugated isotype-specific goat anti-mouse second reagent (Southern Biotechnology Associated, Birmingham, AL). Samples were analyzed by one- or two-color cytofluorimetric analysis (FACSCalibur Becton Dickinson & Co., Mountain View, CA) as previously described (33).

Transient tranfections

HEK-293T cells (4×10^5/plate) were transfected with plasmids coding for the following molecules: KIR2DL1, KIR2DL2, KIR2DL3, KIR2DS1, KIR2DS2, KIR2DS4, KIR3DL1 and KIR3DL2 using FuGene-6 reagent. After 48 h, transfected cells were stained with ECM41, GL183, EB6b, DF200, FES172, Z27, Q66 and AZ158 mAbs followed by isotype-specific PE-conjugated goat anti-mouse second reagent and analyzed by flow cytometry using a FACSort (Becton Dickinson).

Amplification and sequencing of KIR transcripts

Total RNA samples were extracted using peqGOLD RNAPure (Erlangen, Germany) and oligo (dT)-primed cDNA were prepared by standard techniques. The coding primer Ig3-up 5′-CATgTYgCTCAYggTCgTC was used in combination with the reverse primer E: 5′-gTTCCgYgTACACgATgA or C: 5′-AAAACACAgTgATCCAATTA to amplify inhibitory or activating KIR transcripts respectively (30 sec at 95°C, 30 sec at 60°C and 30 sec at 72°C). All the amplification products were purified from gel, and subcloned into pcDNA3.1/V5/His-TOPO vector using the Eukaryotic-TOPO-TA cloning kit (Invitrogen, Carlsbad, CA). To identify the KIR2D expressed in the analyzed samples, at least 15 independent clones were sequenced using d-Rhodamine Terminator Cycle Sequencing kit and a 377 ABI automatic sequencer (Perkin Elmer-Applied Biosystems).

Cytolytic assays

NK cells were tested for cytolytic activity in a 4 h ^{51}Cr-release assay either in the absence or presence of the indicated mAbs. The $Fc\gamma R^+$ P815 (murine mastocytoma) target cell line was used for redirected killing experiments. Other target cells used in these studies were represented by the following B-LCL human cell lines: HLA-class I^- 721.221 cell line either untransfected or transfected with various classical HLA-class I alleles; EBV-24 (obtained in our lab) and KOSE (a kind gift from G.B. Ferrara, Genova, Italy).

The concentrations of the various mAbs were 10 μg/ml for the masking experiments and 0.5 μg/ml for the redirected killing experiments. The E/T ratios are indicated in the text.

Results

ECM41 mAb specifically recognizes KIR2DL3

Mice were immunized with a polyclonal human NK cell line derived from a healthy individual (donor 1) and after fusion, the hybridoma supernatants were first selected by cytofluorimetric analysis on the basis of their ability to stain NK cell subpopulations within the immunizing NK cell line. The reactivity of the selected mAbs was then compared by single and double fluorescence to that of various KIR-specific mAbs including: EB6b (anti-KIR2DL1 and KIR2DS1), GL183 (anti-KIR2DL2, KIR2DL3 and KIR2DS2), FES172 (anti-KIR2DS4), Z27 (anti-KIR3DL1), Q66 (anti-KIR3DL2). By this approach we could identify a mAb, termed ECM41 (IgM) that displayed a specificity similar to that of the GL183 mAb. Indeed, as shown in Fig. 1 (donor 1), double fluorescence cytofluorimetric analysis indicated that the two mAbs marked precisely the same NK cell subset and that all double fluorescent cells were distributed along a diagonal, thus suggesting that the two mAbs were directed to the same surface molecule. However, the analysis of NK cells derived from a large panel of donors revealed that only in ~30% of the cases did the reactivity of the two mAbs overlap as in donor 1. Indeed, in the remaining 60% of the cases, ECM41 mAb stained only a fraction of the GL183$^+$ cells (Fig. 1, donor 2), while in rare cases (10%) ECM41 mAb did not react with the GL183$^+$ cell population analyzed (Fig. 1, donor 3). Importantly, in no instances was ECM41 mAb found to react with GL183$^-$ NK cells. Furthermore, the reactivity of ECM41 mAb did not correlate with any of the other KIR-specific mAbs mentioned above. Therefore the ECM41 mAb appeared to selectively stain a subset of the GL183$^+$ NK cells, thus suggesting a possible reactivity with only one (or two) of the molecules recognized by the GL183 mAb.

To address this possibility we analyzed the reactivity of ECM41 mAb on HEK-293T cells transiently transfected with plasmids coding for KIR2DL2, KIR2DL3 or KIR2DS2 (i.e. KIRs stained by conventional GL183 mAb). As shown in Fig. 2, ECM41 mAb selectively stained KIR2DL3$^+$-transfected cells while, as expected, the GL183 mAb brightly stained all three cell transfectants. The analysis was then extended to HEK-293T cells transfected with one or other of the remaining KIRs whose surface expression could be detected by available specific mAbs. These included KIR2DL1, KIR2DS1, KIR2DS4,

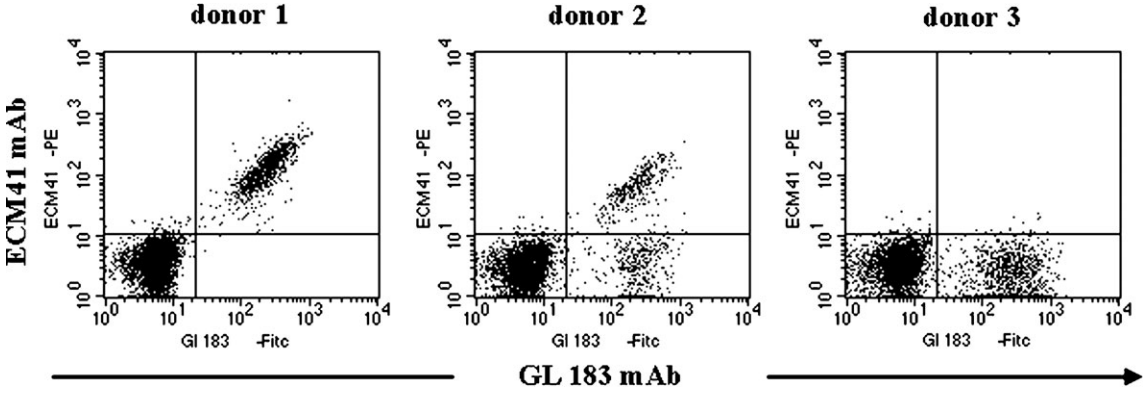

Fig. 1. Cytofluorimetric analysis of ECM41 and GL183-reactive molecules on polyclonal NK cells. Polyclonal NK cell lines derived from representative donors were stained with the indicated mAbs followed by FITC- or PE-conjugated isotype-specific goat anti-mouse second reagents. Similar results were obtained by the analysis of freshly isolated NK cells from the same donors. These results are representative of more than 20 donors analyzed.

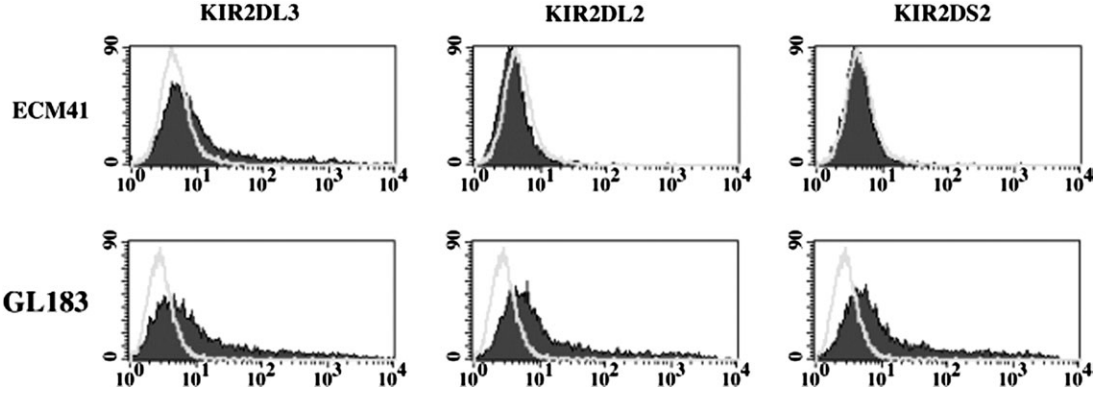

Fig. 2. ECM41 mAb specifically recognizes KIR2DL3 molecule. HEK-293T cells transiently transfected with the indicated constructs were stained with GL183 or ECM41 mAbs followed by PE-conjugated goat anti-mouse isotype specific second reagents. Gray profiles indicate staining with second reagent alone. Cells were analyzed by cytofluorimetry.

KIR3DL1 and KIR3DL2. As summarized in Table 1, none of these transfectants was stained by ECM41 mAb.

These results demonstrate that ECM41 mAb represents a unique reagent able to discriminate KIR2DL3 from other KIRs, and in particular from other GL183 mAb-reactive KIRs (KIR2DL2 and KIR2DS2). Based on this finding, the profiles shown in Fig. 1 would be representative of the distribution of distinct GL183-reactive KIRs in NK cells derived from different donors. In particular, the GL183$^+$ECM41$^+$ cells (observed in donors 1 and 2) correspond to those expressing KIR2DL3 while GL183$^+$ECM41$^-$ cells (observed in donors 2 and 3) represent those expressing KIR2DL2 and/or KIR2DS2 but not KIR2DL3.

KIR2DL2 and KIR2DL3 recognize the same HLA-C alleles

By the use of soluble KIR molecules, KIR2DL2 and KIR2DL3 have been shown to react with a defined cluster of HLA-C alleles identified by the presence of the Asn80 residue (HLA-C^{Asn80} alleles) (25,31,34,35). Here we analyzed the HLA specificity of single NK clones expressing one or other of the two distinct GL183 mAb-reactive inhibitory KIRs. To this end we selected NK cell clones characterized by the GL183$^+$ECM41$^+$ and the GL183$^+$ECM41$^-$ phenotypes and analyzed their KIR transcripts. The sequences of the RT–PCR products obtained using primer pairs able to amplify the inhibitory KIR revealed that some of GL183$^+$ECM41$^+$ clones were KIR2DL3$^+$KIR2DL2$^-$, while others were KIR2DL3$^+$KIR2DL2$^+$ (not shown). Two NK clones were further selected on the basis of their phenotypes and utilized in functional analysis. Clone DP1 expressed the GL183$^+$ECM41$^+$ (KIR2DL3$^+$KIR2DL2$^-$) phenotype while clone L38 expressed the GL183$^+$ECM41$^-$ (KIR2DL3$^-$KIR2DL2$^+$) phenotype. Both the clones were NKG2A$^-$ and did not react with other available KIR-specific mAbs. DP1 and L38 were analyzed for cytolytic activity against a panel of B EBV-transformed target cells expressing HLA-C^{Asn80} alleles. These targets included: the HLA class I negative 221 cell line transfected with HLA-Cw3 or -Cw8 alleles and the homozygous cell lines EBV24 and EBV-KOSE expressing the HLA-Cw7 and -Cw12 alleles, respectively. As shown in Fig. 3, both NK clones efficiently killed untransfected 221 cells while sparing all the other target cells. However, the HLA-C positive targets were lysed by both

Table 1. Specificity of different anti-KIR mAb

KIR-specific mAbs	KIR2DL3	KIR2DL2	KIR2DS2	KIR2DL1	KIR2DS1	KIR2DS4	KIR3DL1	KIR3DL2
ECM 41	+	–	–	–	–	–	–	–
GL 183	+	+	+	–	–	–	–	–
EB6b	–	–	–	+	+	–	–	–
DF 200	+	+	+	+	+	–	–	–
FES 172	–	–	–	–	–	+	–	–
Z27	–	–	–	–	–	–	+	–
Q66	–	–	–	–	–	–	–	+
AZ 158	–	–	–	–	–	–	+	+

The mAbs shown in the first column were analyzed for their ability to stain the HEK-293T cell line transfected with one or another of the indicated KIR.

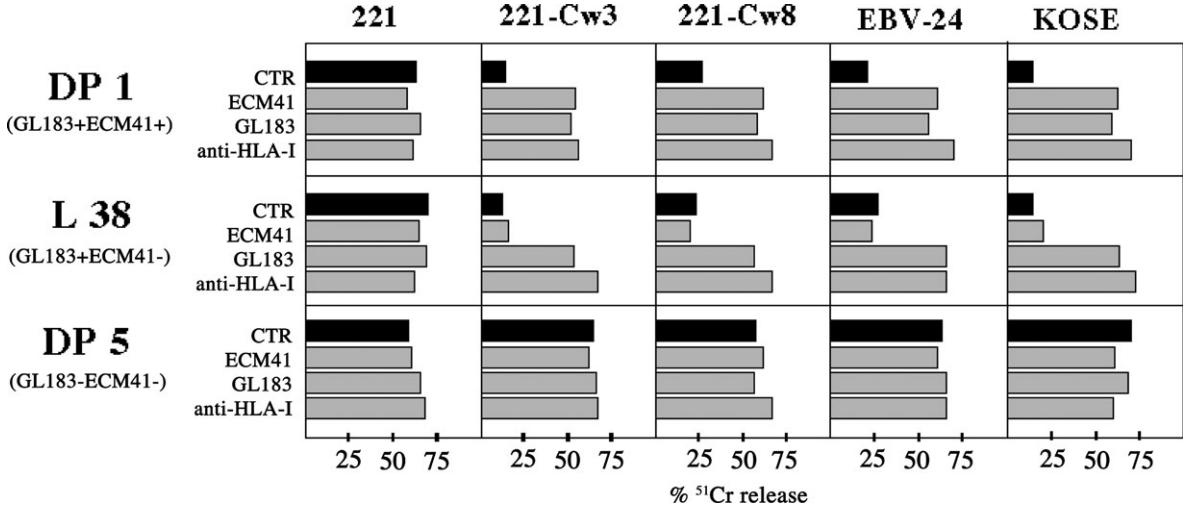

Fig. 3. KIR2DL2$^+$ and KIR2DL3$^+$ NK clones recognize the HLA-C^{Asn80} alleles. The following NK cell clones, DP1 (KIR2DL2$^-$KIR2DL3$^+$), L38 (KIR2DL2$^+$KIR2DL3$^-$) and DP5 (KIR2DL2$^-$KIR2DL3$^-$), were analyzed in a cytolytic assay against the HLA class I negative (221) or HLA-C^{Asn80} positive (221-Cw3, 221-Cw8, EBV24 and KOSE) targets. The reactivity with GL183 and ECM41 mAb (in brackets) for each clone is also shown. The experiments (representative of five independent tests) were carried out either in the absence (CTR) or in the presence of the indicated mAbs (anti-HLA class I mAb is represented by the A6/136 mAb). E/T ratio: 3/1.

clones in the presence of appropriate anti-HLA class I specific mAb. These data indicate that both KIR2DL3 (expressed by DP1) and KIR2DL2 (expressed by L38) were able to recognize HLA-Cw3, -w7, -w8 and -w12 alleles. This was further confirmed by the fact that mAb-mediated masking of either KIR2DL2 (by addition of GL183 mAb on clone L38) or KIR2DL3 (by addition of either ECM41 or GL183 mAb on clone DP1) efficiently reconstituted lysis of the above HLA-C-protected target cells. These results are representative of >20 clones analyzed. In these experiments, control NK clones lacking KIR2DL2/3 such as clone DP5 (NKG2A$^-$KIR2DL2/3$^-$KIR2DS2$^-$KIR2DL1$^+$) displayed cytolytic activity against both transfected and untransfected 221 cells, as well as against the two homozygous cell lines.

Although not shown, both GL183$^+$ NK clones (L38 and DP1) efficiently killed 221 cells transfected with HLA class I alleles different from those belonging to the HLA-C^{Asn80} group, thus confirming that both KIR2DL2 and KIR2DL3 confer the same HLA specificity to NK cells.

Analysis of NK clones co-expressing the inhibitory and the activating forms of GL183 mAb reactive molecules

As mentioned above, available KIR2D-specific mAbs cannot discriminate between the activating and the inhibitory form of a given KIR. Indeed, to determine the precise type of KIR expressed at the surface of a given NK cell clone, beyond the cytofluorimetric analysis, a direct functional analysis is generally required. Thus KIR-specific mAb (of the IgG isotype) can be assessed in redirected killing assay for their ability to modulate the spontaneous NK-mediated killing of FcγR$^+$ P815 murine target cells. In this type of assay, mAbs directed to triggering receptors induce increments of cytotoxicity whereas mAbs to inhibitory receptors reduce the spontaneous NK-mediated lysis of P815. The use of a mAb specific for both the activating and the inhibitory form of a given receptor (such as the GL183 mAb) does not allow a precise assessment of the functional nature of the KIR recognized, especially when these molecules are co-expressed at the cell surface. Indeed, in this case, since in NK cells the inhibitory signals generally suppress the activating ones, the simultaneous engagement of the two receptors might be expected to result in inhibition of cytotoxicity. The availability of the ECM41 mAb, however, may offer the possibility of overcoming this problem. Indeed in a 'redirected killing assay' the use of ECM41 mAb, which is of the IgM isotype and therefore unable to bind to Fc-receptors expressed on P815, would provide a selective masking of KIR2DL3 without induction of negative signals. In the case of clones co-expressing KIR2DL3 and KIR2DS2, the ECM41 mAb would then compete with GL183 mAb for binding to KIR2DL3 and, as a result of this competition, the GL183 mAb would prevalently react with KIR2DS2. The effect of the GL183 mAb alone or in combination with the ECM41 mAb was then analyzed in a redirected killing assay on a panel of >50 NK cell clones expressing the GL183$^+$ECM41$^+$ phenotype. As expected, the NK-mediated lysis of most of these clones was inhibited by the GL183 mAb used alone. Importantly, this inhibition was weakened (but not reverted) when GL183 was used in combination with the ECM41 mAb (see the representative clone P8/2 in Fig. 4A). In a few NK clones however (that were named 'ECM41/Act' NK clones), the addition of ECM41 mAb completely abrogated the inhibitory effect of the GL183 mAb, resulting in strong increase of cytotoxicity (see the representative clone P8/6 in Fig. 4A and five additional clones in Fig. 4B). In these clones, the triggering effect induced by the combination of ECM41 and GL183 mAbs was comparable in magnitude to that induced by GL183 mAb alone on a clone expressing KIR2DS2 but not KIR2DL2/3 (clone P19/100, Fig. 4A). The effects mediated by ECM41 mAb were specific, since (i) they could not be obtained by other mAbs of the IgM isotype (including the A6/220 mAb, specific for the CD56 molecule, and the Y249 mAb that displays the same reactivity of the GL183 mAb) (data not shown); and (ii) when added alone, ECM41 mAb didn't affect the spontaneous NK-mediated killing of P815 target cells (Fig. 4A).

Thus, while the results obtained on clone P8/2 can be explained by the competition of ECM41 and GL183 mAb for KIR2DL3, the case of clone P8/6 suggested that both KIR2DL3 and KIR2DS2 might have been expressed at the cell surface. This was confirmed by molecular analysis of the KIR transcripts present in clones P8/2, P8/6 and P19/100. Indeed RT–PCR analysis revealed that the P8/2 clone was KIR2DL2$^-$KIR2DL3$^+$KIR2DS2$^-$, the P8/6 clone was KIR2DL2$^-$KIR2DL3$^+$KIR2DS2$^+$ while the P19/100 clone was KIR2DL2$^-$KIR2DL3$^-$KIR2DS2$^+$.

In the 'ECM41/Act' NK clones (that should express the KIR2DL2$^-$KIR2DL3$^+$KIR2DS2$^+$ phenotype) the inhibitory effect of GL183 mAb alone was variable, ranging from a complete to a hardly detectable inhibition of the spontaneous NK-mediated lysis (Fig. 4B). This is explained by the fact that in each clone the cytolytic outcome resulting from the engagement of GL183-reactive KIRs depends on the balance between the signals delivered by inhibitory vs activating KIRs.

The mAb-mediated engagement of KIR2DL3 resulted in strong inhibition of NK cell activation induced simultaneously via different triggering NK receptors. Indeed, as shown in clone P8/6 (Fig. 4A), the simultaneous addition of GL183 mAb (that in this clone interacts with both KIR2DL3 and KIR2DS2) and of anti-NKp46 mAb resulted in inhibitory signals capable of abrogating lysis.

Discussion

In this study, by the generation of a novel KIR-specific mAb (ECM41), we could compare the cell surface distribution and HLA specificity of two inhibitory KIRs (KIR2DL2 and KIR2DL3) that were previously identified but not distinguished by the GL183 mAb. Remarkably, this novel reagent, which is specific for KIR2DL3, also allows the functional dissection of GL183-reactive activating and inhibitory receptors co-expressed at the cell surface of given NK cells.

Previous studies indicated that the HLA-C alleles bearing residues Ser77–Asn80 (initially identified as 'group 1' alleles) are recognized by inhibitory KIRs identified by the GL183 mAb (15,33,36–38). These receptors, that were originally termed p58.2, consist of two distinct gene products termed KIR2DL2 and KIR2DL3. Their HLA specificity was previously assessed through the combined use of either soluble KIR molecules or GL183$^+$ NK clones and HLA class I transfected cell lines.

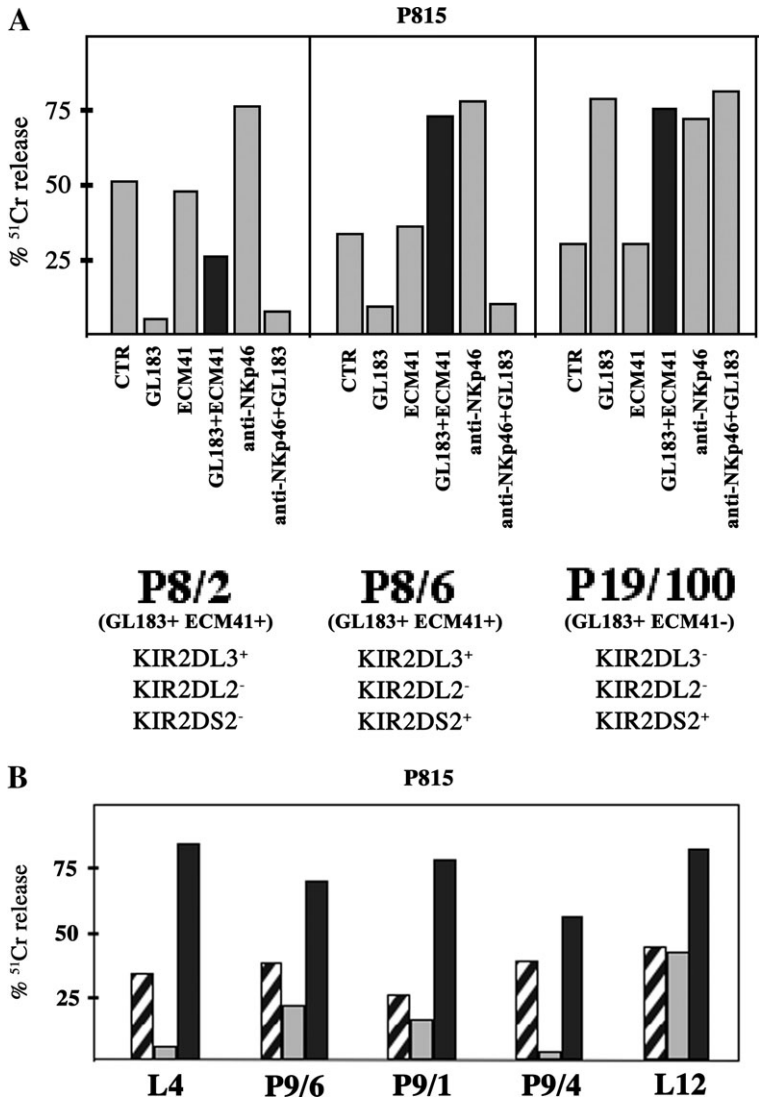

Fig. 4. The combined use of ECM41 and GL183 mAbs in a redirected killing assay allows the identification of KIR2DL3$^+$KIR2DS2$^+$ and KIR2DL3$^+$KIR2DS2$^-$ NK clones. (A) The cytolytic activity against P815 target cells of three representative NK clones displaying the indicated KIR phenotype (assessed by RT–PCR analysis) is shown. The reactivity with GL183 and ECM41 mAb (in brackets) for each clone is also indicated. These two mAb were added alone or in combination (black bars) in the cytolytic test. GL183 mAb was also added in combination with anti-NKp46 mAb (BAB281) in order to assess the ability of KIR2DL3 to block simultaneously two different triggering signals (induced by KIR2DS2 and NKp46). (B) The effect of GL183 and ECM41 mAb was analyzed on the cytolytic activity mediated by different KIR2DL3$^+$KIR2DS2$^+$ NK clones. Striped bars: spontaneous lysis. Gray bars: lysis in the presence of GL183 mAb. Black bars: lysis in the presence of GL183 mAb + ECM41 mAb. All the experiments were performed at an E:T ratio of 3:1. These results are representative of five independent experiments.

However, the functional experiments aimed to compare KIR2DL2$^+$ and KIR2DL3$^+$ NK clones were impaired by the lack of mAbs able to discriminate between the two different KIRs. The novel ECM41 mAb specifically recognizes KIR2DL3 but not KIR2DL2, and therefore allows the identification of GL183$^+$ KIR2DL3$^+$ and GL183$^+$KIR2DL3$^-$ NK clones. The analysis of the two types of NK clones confirmed that indeed both KIR2DL2 and KIR2DL3 recognize essentially the same pattern of HLA class I alleles. This result is nevertheless important, since the assessment of the precise HLA specificity of a given KIR may represent a crucial requirement in case the NK cells should be employed in hematopoietic transplants as proposed by Ruggeri *et al.* (39,40).

In this study, we also evaluated the effect of the simultaneous engagement of activating and inhibitory KIRs reacting with GL183 mAb. Our results clearly indicate the occurrence of NK clones expressing both KIR2DS and KIR2DL at the cell surface. In these clones, the effect mediated by the inhibitory KIR overrides that of the activating one. Indeed, in clones expressing the KIR2DS2$^+$KIR2DL3$^+$ phenotype, the GL183 mAb-dependent KIR2DS2 cross-linking resulted in an enhancement of cytolytic activity only after masking of the inhibitory KIR2DL3 by the ECM41 mAb. A recent study by Warren *et al.* suggested that KIR2DS2 and KIR2DL2/3, also when co-expressed, may exert their function independently (41). However, the above experiments were carried out using

mAbs unable to discriminate the two KIR types (for example GL183) and therefore were unable to define the precise KIR phenotype of the NK cell population under study.

Our analysis of a large panel of KIR2DS2$^+$KIR2DL3$^+$ NK clones also revealed a certain variability in the activity of the two KIR types. Indeed, depending on the clones analyzed, the effect resulting from the simultaneous engagement of both receptors by the GL183 mAb ranged between a strong to a hardly detectable inhibition of the spontaneous NK-mediated lysis of P815 target cells (Fig. 4B). This suggests that the behavior of a given NK cell expressing both KIR2DS and KIR2DL during the interaction with a cell target may result (at least in part) from the balance of the signals delivered by the two KIRs displaying opposite functions. Although both the inhibitory and the activating KIRs are referred to as recognizing HLA class I molecules, important differences exist. For example, the activating KIRs, different from the inhibitory ones, appear to recognize HLA class I molecules with low efficiency. We do not actually know whether the presence of abnormal (viral?) peptides might strengthen the KIR2DS/HLA class I interaction or whether virus-encoded HLA homologues would represent the actual targets for the activating KIRs. Recent reports suggest an involvement of the MHC-specific activating NK receptors in the control of viral infections (42,43). In this context, the co-expression of both inhibitory and activating forms of a given KIR and their functional interactions may be seen as a mechanism to 'sense' a differential loading of abnormal vs normal peptides on a given HLA class I allele or a differential expression of 'homologous' vs autologous HLA class I molecules.

Acknowledgements

This work was supported by grants awarded by Associazione Italiana per la Ricerca sul Cancro (A.I.R.C.), Istituto Superiore di Sanità (I.S.S.), Ministero della Sanità, Ministero dell'Università e della Ricerca Scientifica e Tecnologica (M.U.R.S.T.). Also the financial support of Fondazione Compagnia di San Paolo, Torino, Italy, is gratefully acknowledged.

Abbreviations

B-LCL	B-lymphoblastoid cell line
FcγR	Fcγ-receptor
KARAP	killer cell-activating receptors-associated polypeptides
KIR	killer Ig-like receptors
NK	natural killer

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